

SPORTIVE LEMUR DIVERSITY AT MANANARA-NORD BIOSPHERE RESERVE, MADAGASCAR

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ABSTRACT

Molecular genetic sequence variation among the sportive lemurs (genus *Lepilemur*) of Madagascar was investigated utilizing mitochondrial DNA (mtDNA) sequence data (ca. 3,000 base pairs). We infer *Lepilemur* phylogenetic relationships based on the topology of mtDNA trees generated with 225 individuals from 24 currently recognized species of the genus from 43 field sites, along with a previously undefined taxon from Mananara-Nord Biosphere Reserve. We formally describe this new species based on genetic analyses and biogeographic information (including the recent Inter-River-Systems model), supplemented with morphological data.

Key words: biogeography, *Lepilemur*, Madagascar, Mananara-Nord Biosphere Reserve, phylogenetics, sportive lemur

INTRODUCTION

Madagascar's remarkable species diversity and high levels of endemism are persistently under threat from anthropogenic pressures (Mittermeier et al. 2006; Harper et al. 2007). Consequently, the island has been ranked among the world's most important biodiversity hotspots, underscoring the need for coordinated conservation efforts (Myers 2000; Groombridge and Jenkins 2002). While most of the Malagasy fauna is susceptible to extinction risk, lemurs are especially vulnerable due to their relatively small and often fragmented geographic ranges (Jernvall and Wright 1998). As a result, lemurs are protected under the Convention on International Trade in Endangered Species (CITES) and many species are red-listed as Critically Endangered, Endangered or Vulnerable by the IUCN SSC (IUCN 2008). Many species are still data deficient (IUCN 2008), and their number is likely to increase due to the recent rapid expansion in the number of recognized species (e.g., Andriaholinirina et al. 2006; Louis et al. 2006b; Rabarivola et al. 2006; Craul et al. 2007, 2008; Lei et al. 2008). In addition, the description of new species often reduces the geographic range of traditionally recognized taxa (Louis et al. 2006b). Thus, frequent re-evaluation of the conservation status of all lemur species is necessary, using newly available informa-

tion on taxonomy, biogeography, ecology, ethology, and current threats.

Among the most widely distributed lemur groups, the sportive lemurs (genus *Lepilemur*) are medium-sized nocturnal lemurs that were originally thought to consist of only two species, *L. mustelinus* from the eastern rainforests and *L. ruficaudatus* from the western and southern dry forests of the island (Schwarz 1931; Hill 1953). Recent investigations using molecular genetic, cytogenetic, and morphological data have greatly expanded the diversity of this genus (Louis et al. 2006b; Rabarivola et al. 2006; Craul et al. 2007, 2008; Lei et al. 2008). These developments in *Lepilemur* taxonomy are a salient example of the need for verifying unique diversity in order to assess the conservation status of a group. Currently, there is one vulnerable, one endangered, and one critically endangered *Lepilemur* species, while 21 of the listed species (most of them recently described) remain data deficient (IUCN 2008).

Northeastern Madagascar is one region where sportive lemur taxonomy is currently in revision. *L. mustelinus* was formerly the only recognized taxon between the Bemarivo and Mangoro Rivers (Petter et

al. 1977; Tattersall 1982). More recently, two previously undefined species have been described from this region. Louis et al. (2006b) described *L. seali* from Anjanaharibe-Sud Special Reserve, while Lei et al. (2008) introduced *L. scottorum* from the Masoala Peninsula (Fig. 1). Furthermore, Craul et al. (2008) extended the distribution of *L. seali* south of the Antainambalana River. Louis et al. (2006b) assigned the sportive lemur from Mananara-Nord Biosphere Reserve (MNBR) to *L. seali* although the available molecular data suggested that this population would eventually be described, pending further field studies. With only a single representative from MNBR available, Lei et al. (2008) established it as an undefined species, *Lepilemur species nova* #2. By incorporating sequence data from Louis et al. (2006b), Craul et al. (2008) subsequently reconfirmed the uniqueness of the population at MNBR.

Three biogeographic models have been proposed based on different relative contributions of factors including large rivers (>50 m wide at 20 km inland), retreat dispersion watersheds, and topographical barriers, i.e. mountains (Martin 1995; Wilmé et al. 2006; Craul et al. 2007; Olivieri et al. 2007). In Olivieri et al. (2007) and Craul et al. (2008) the authors presented biogeographic models in which “centers of endemism” were defined based on the isolation effects of paired rivers, or Inter-River-System (IRS; Fig. 1). During the course of several biogeographic revisions of northern and northwestern Madagascar, the number of IRS has increased from four (Martin 1995), to five (Wilmé et al. 2006), and to nine (Craul et al. 2008). In Craul et al. (2008), an initial IRS model was presented to delineate the ranges of the sportive lemurs of northeastern Madagascar. We aim to add to the baseline understanding of the diversity in the greater Antongil Bay region, which includes Anjanaharibe-Sud Special Reserve, Masoala National Park, Makira Forest, Mananara-Nord Biosphere Reserve, and adjacent habitats. By revising the known biodiversity of sportive lemurs for this region, we present an amended IRS model to one of the largest remaining tracts of intact forest in Madagascar (Fig. 1).

Historically, the Biological Species Concept (BSC), emphasizing reproductive isolation, has been the most common approach to define species (Mayr 1942). However, when a putative species is geographically isolated from closely related species, this concept is difficult to implement. The Phylogenetic Species Concept (PSC) employs a cladistic perspective, incorporating evolutionary patterns of ancestry and descent as it defines species operationally as the smallest diagnosable, distinct cluster of individuals (Cracraft 1983; Wheeler and Platnick 2000; Groves 2001a, b). It is also useful in defining conservation units (Vogler and DeSalle 1994). In this paper, we present a taxonomic analysis of the genus *Lepilemur* using the PSC, including a formal description of a new species from MNBR.

As previously discussed in Andriantompohavana et al. (2006), Louis et al. (2006a, b) and Thalmann and Geissmann (2005), the utilization of whole vouchers as the designated holotype for a new species is not a prerequisite for species descriptions; opportunistic collections can later supplement morphological, vocalization, and/or molecular data in combination with curated blood and/or tissue samples (Jones et al. 2005). The sportive lemurs are a prime candidate for this methodology because the highly folivorous dietary requirements of this group currently preclude any attempts to curate “live vouchers” (Thalmann and Geissmann 2005). Total genomic DNA for the three paratype specimens is currently curated at the Museum of Texas Tech University (TK125726; TK125727; TK125728). Additionally, an electronic database that contains all *Lepilemur* field data and photographs, including data for the paratype specimens, is curated at the Museum of Texas Tech University. The database is stored in the Type Specimen Collection in multiple media formats. This collection of field data and photographs, as well as additional tables and figures, is also available online at the website of Omaha’s Henry Doorly Zoo. See Appendices I-III for a directory of appropriate website addresses.

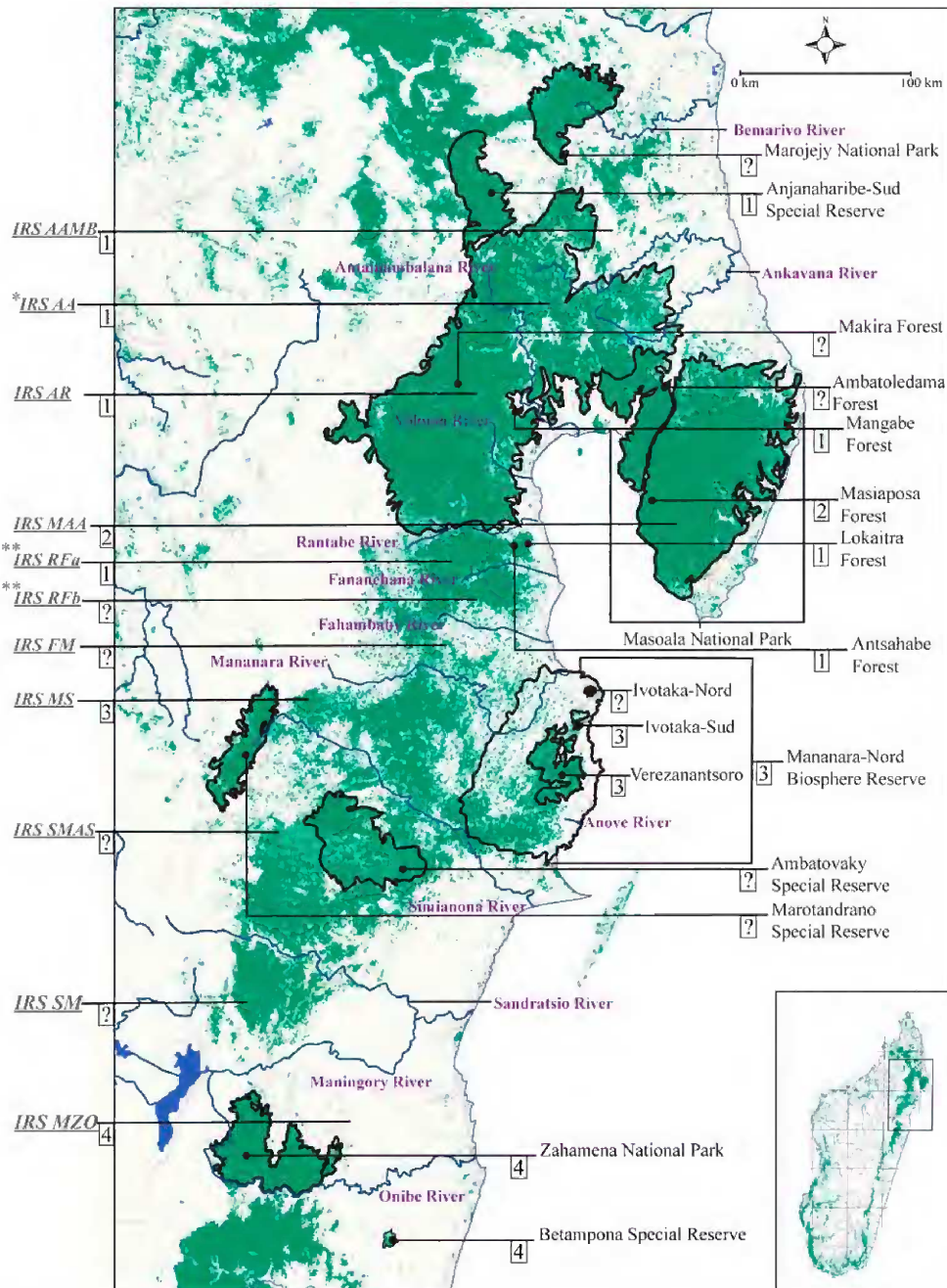


Figure 1. Map of northeastern Madagascar. This regional map features the potential boundaries and forest tracts for the sportive lemurs (genus *Lepilemur*) in northeastern Madagascar. Samples collected in the Ivotaka-Sud and Verezanantsoro parcels of Mananara-Nord Biosphere Reserve, Madagascar, were evaluated, along with sequence data from Louis et al. (2006b) and Lei et al. (2008). The Inter-River-System (IRS) model was adapted from Craul et al. (2008). IRS A and IRS RF from Craul et al. (2008) were revised to IRS AA and IRS RFa and RFb, respectively. IRS SMAS, IRS SM, and IRS MZO were inserted to define the potential boundaries for *L. species nova* #2 (Lei et al. 2008) and *L. mustelinus*. IRS AAMB was inserted to define the potential northern boundary for *L. seali*. IRS MAA was inserted to define the potential boundaries for *L. scottorum*. The boxed numbers found below the horizontal lines indicate what sportive lemur is designated for that site or IRS as follows: 1 specifies *L. seali*; 2 specifies *L. scottorum*; 3 specifies *L. sp. nova* #2; 4 specifies *L. mustelinus*; and ? specifies undefined species or data unavailable.

METHODS

Sampling.—All lemurs investigated in this study were wild-caught, free-ranging individuals immobilized with a CO₂ projection rifle or blowgun with 10 mg/kg of Telazol (Fort Dodge Animal Health; Overland Park, Kansas; Fig. 1; Table 1). Four 2.0 mm biopsies and 1.0 cc per kilogram of whole blood were collected from each sedated animal and immediately stored in room temperature storage buffer (Longmire et al. 1992). A HomeAgain microchip (Schering-Plough Veterinary Corp.; Kenilworth, New Jersey) was placed subcutaneously between the scapulae of each lemur (Appendix I(a)). This procedure was used to field-catalog each animal with a unique recognition code in order to re-identify all captured individuals during any future immobilizations. In addition, morphometric measurements were taken. For presentation purposes, we present the weight, head crown, body length, and tail length in this publication following the guidelines of Smith and Jungers (1997; Appendices I(a-b)). Field data, including all measurements and e-voucher photographs, are available in Appendix I(b), Louis et al. (2006b), and Lei et al. (2008).

Data Collection.—We recorded the location of all immobilized lemurs using a global positioning system (GPS; Appendix I(a-b)). Genomic DNA was extracted from samples using a phenol-chloroform/isoamyl extraction (Sambrook et al. 1989). We recorded the location of all immobilized lemurs using a global positioning system (GPS; Appendix I(a-b)). From these samples, the following regions of mtDNA were amplified: the displacement loop or control region (D-loop; Baker et al. 1993; Wyner et al. 1999), a fragment of the cytochrome oxidase subunit III gene (COIII), NADH-dehydrogenase subunits 3, 4L, and 4 (ND3, ND4L, and ND4), as well as the tRNA^{Gly}, tRNA^{Arg}, tRNA^{His}, tRNA^{Ser}, and partial tRNA^{Leu} genes (subsequently referred to as the PAST fragment; Louis et al. 2006b). The accessioned sequences of *L. seali* and *L. mittermeieri* of Craul et al. (2008) and Rabarivola et al. (2006), respectively, were not compatible with this data set so were not utilized in this study. Although available as accession sequences in GenBank, we would have had to truncate and eliminate two-thirds of our generated sequence data to include their *L. seali* and *L. mittermeieri* accession fragments in these analyses. Using 50 nanograms of genomic DNA, the D-loop (555

bp) and the PAST (2,378 bp) fragments were amplified using the following conditions: 94°C for 30s, 47°C for 45s, and 72°C for 45s for 34 cycles. Since potential nuclear insertions or mitochondrial pseudogenes within the nuclear genome can be amplified inadvertently, we minimized the likelihood by amplifying both mitochondrial DNA regions as intersecting or overlapping segments and confirming these segments with the degenerate oligonucleotide-primed PCR methodology (Telenius et al. 1992; Zhang and Hewitt 1996; Louis et al. 2006b). The samples were electrophoresed on a 1.2% agarose gel to verify the PCR product and purified with Exonuclease I and Shrimp Alkaline Phosphatase (EXOSAP; Silva et al. 2001).

The purified products were cycle-sequenced using a BigDye terminator sequencing kit (Applied Biosystems; Foster City, California). The sequences were analyzed by capillary electrophoresis with an Applied Biosystems Prism 3130 genetic analyzer. The PCR and sequencing primer suite from Louis et al. (2006b) and Lei et al. (2008) were used to generate the D-loop and PAST fragment sequences. The sequence fragments were aligned to generate a consensus sequence using Sequencher 4.8 (Gene Codes Corporation; Ann Arbor, Michigan), and the consensus sequences were aligned using ClustalX 1.83 (Thompson et al. 1997). All aligned sequences are available from the first author upon request. All sequences have been deposited in GenBank, and the sequence data and information are available from the referenced accession numbers (Table 1; Appendix I(a); Louis et al. 2006b; Lei et al. 2008).

Phylogenetic Analysis.—Maximum likelihood (ML) analyses for the D-loop and PAST fragment sequence data were performed under the GTRCAT algorithm implemented in the parallel Message Passing Interface (MPI) version of RAXML-VI-HPC (Stamatakis 2006; software available at <http://icwww.epfl.ch/~stamatak>). The best-scoring ML-trees were searched and saved in PAUP* 4.0b10 (Swofford 2001). Bayesian inference analyses of the D-loop and PAST fragment sequence data were conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). A Markov Chain Monte Carlo (MCMC) run with four simultaneous chains and 1,000,000 generations was performed.

Table 1. Samples (225 *Lepilemur* and 29 outgroups total) used in the present genetic study and taxonomic revision of the genus *Lepilemur*. TK Number^a is referenced voucher curated at the Museum of Texas Tech University. Mitochondrial DNA sequence data for PAST (*Pastorini Fragment*), and D-loop (D-loop or control region) for each sample are available from GenBank under the listed accession numbers^b.

| Catalog Number | TK Number ^a | Site | Original Species Designation | Current Species Designation | D loop ^b | PAST ^b |
|----------------|------------------------|------------------------------|-------------------------------------|-------------------------------------|---------------------|-------------------|
| NARA4.20 | TK125726 | Mananara-Nord (Ivontaka-Sud) | <i>Lepilemur mustelinus</i> | <i>Lepilemur hollandorum</i> | DQ529552 | DQ529694 |
| NARA8.5 | TK125727 | Mananara-Nord (Ivontaka-Sud) | <i>Lepilemur mustelinus</i> | <i>Lepilemur hollandorum</i> | EU779982 | EU779966 |
| NARA8.7 | TK125728 | Mananara-Nord (Ivontaka-Sud) | <i>Lepilemur mustelinus</i> | <i>Lepilemur hollandorum</i> | EU779983 | EU779967 |
| NARA8.8 | | Mananara-Nord (Ivontaka-Sud) | <i>Lepilemur mustelinus</i> | <i>Lepilemur hollandorum</i> | EU779984 | EU779968 |
| NARA8.11 | | Mananara-Nord (Ivontaka-Sud) | <i>Lepilemur mustelinus</i> | <i>Lepilemur hollandorum</i> | EU779985 | EU779969 |
| NARA8.12 | | Mananara-Nord (Ivontaka-Sud) | <i>Lepilemur mustelinus</i> | <i>Lepilemur hollandorum</i> | EU779986 | EU779970 |
| BIBO7.1 | | Ambodimahabibo | <i>Lepilemur edwardsi</i> | <i>Lepilemur otto</i> | EU779987 | EU779971 |
| BIBO7.3 | | Ambodimahabibo | <i>Lepilemur edwardsi</i> | <i>Lepilemur otto</i> | EU779988 | EU779972 |
| BIBO7.6 | | Ambodimahabibo | <i>Lepilemur edwardsi</i> | <i>Lepilemur otto</i> | EU779989 | EU779973 |
| BIBO7.7 | | Ambodimahabibo | <i>Lepilemur edwardsi</i> | <i>Lepilemur otto</i> | EU779990 | EU779974 |
| TVY7.23 | | Ambatovy | <i>Allocebus trichotis</i> | <i>Allocebus trichotis</i> | EU779975 | EU779959 |
| ZOMB10 | | Zombitse | <i>Phaner furcifer pallescens</i> | <i>Phaner pallescens</i> | EU779976 | EU779960 |
| ANT5.3 | | Antafondro (Ambohimandra) | <i>Mirza coquereli</i> | <i>Mirza zaza</i> | EU779977 | EU779961 |
| ZOMB54 | | Zombitse | <i>Mirza coquereli</i> | <i>Mirza coquereli</i> | EU779978 | EU779962 |
| MER1 | | Analamerana | <i>Eulemur coronatus</i> | <i>Eulemur coronatus</i> | EU779979 | EU779963 |
| HIH6 | | Anjiamangirana | <i>Daubentonia madagascariensis</i> | <i>Daubentonia madagascariensis</i> | EU779980 | EU779964 |
| REN31 | | Berenty | <i>Lemur catta</i> | <i>Lemur catta</i> | EU779981 | EU779965 |

The model of evolution was selected for the ML inferences by using Mrmodeltest 2.2, a modified version of Modeltest 3.6 (Posada and Crandall 1998; Nylander 2004). It was performed with HKY+I+G Model for D-loop and GTR+I+G Model for PAST, for 1,000,000 generations. Every hundredth generation, the tree with the best likelihood score was saved, resulting in 4,000 trees. These were condensed in a majority rule consensus tree using PAUP* 4.0b10 (Swofford 2001), and clade posterior probabilities (PP) were computed. The pattern of sequence evolution was estimated by conducting a minimum spanning network generated with the program NETWORK version 4.500 (Bandelt et al. 1999) and Arlequin version 2.0 (Schneider et al. 2000). MEGA 3.1 (Kumar et al. 2004) was used to calculate uncorrected pairwise distances ('p') and

Kimura distance measures (Kimura 1980) for D-loop and PAST fragments.

We utilized MacClade 3.01 (Maddison and Maddison 1992) and MEGA version 3.1 (Kumar et al. 2004) in a diagnostic search to designate evolutionary significant units (ESU) using population aggregate analysis (PAA) of the D-loop (550 bp) and PAST (2,378 bp) sequence data for genus *Lepilemur* (Davis and Nixon 1992; Louis et al. 2006a, b; Lei et al. 2008). With the sequential addition of each individual without an *a priori* species designation, a PAA distinguishes attributes or apomorphic characters according to the smallest definable unit (Davis and Nixon 1992; Vogler and DeSalle 1994; Groves 2001a, b; Louis et al. 2006b; Lei et al. 2008).

RESULTS

Mitochondrial DNA sequence data were completed for two fragments, D-loop and PAST (ca. 3,000 bp), for 225 individuals representing 24 recognized species of sportive lemurs collected from 43 field sites (Table 1; Louis et al. 2006b; Lei et al. 2008). Due to different mtDNA fragments utilized in Rabarivola et al. (2006), congruent sequence data were not available and thus, *L. mittermeieri* was not included in this study. Based on the phylogenetic inferences of the ML and Bayesian analyses of D-loop and PAST sequence alignments, 24 *Lepilemur* species were represented in 24 distinct and well-supported terminal clades. These terminal clades could be partitioned into four geographic regions (Figs 2-3; Appendices II(a-h)). In general, Section A consists of sportive lemurs from northern and northwestern Madagascar as follows: *L. ankaranensis*, *L. milanoii*, *L. septentrionalis*, *L. tymerlachsoni*, *L. dorsalis*, *L. sahamalazensis*, and *L. ahmansonorum*. Section B is associated with northwestern Madagascar: *L. otto*, *L. edwardsi*, and *L. grewcockorum*. Section C corresponds to southern and west central Madagascar as follows: *L. hubbardorum*, *L. ruficaudatus*, *L. aeeclis*, *L. randrianasoli*, *L. leucopus*, and *L. petteri*. With the exception of *L. microdon*, Section D incorporates the sportive lemurs of eastern Madagascar as follows: *L. mustelinus*, *L. jamesorum*, *L. betsileo*, *L. fleuretae*, *L. wrightae*, *L. seali*, *L. scottorum*, and *L. species nova* #2. Furthermore, all phylogenetic methods support the

uniqueness of the subpopulation, *Lepilemur species nova* #2 from MNBR (Figs. 2-3).

All methods revealed the same phylogenetic proximity between regions and among sportive lemur species, resulting in distinguishable eastern and western clades with the exception of *L. microdon* (Figs. 2-3; Appendices II(a-h)). The phylogenetic association of *L. microdon* to Sections B or C varied according to which mtDNA sequence fragment was analyzed. Based on either phylogenetic method, *L. microdon* clusters with the northwestern sportive lemurs (Section B) for the D-loop sequence fragment. However, for the PAST sequence fragment, this east coast sportive lemur's association shifts to the species located in west central and southern Madagascar (Section C; Figs. 2-3; Appendices (a-h)).

The complete uncorrected 'p' distance and the Kimura two-parameter distance measures for the genus *Lepilemur* are presented for D-loop and PAST fragments in Appendices III(c-d). Values ranged from 7.0% to 10.9% and from 4.2% to 11.0% for D-loop and PAST, respectively, for the three sportive lemur species geographically closest to *L. species nova* #2. The minimum spanning network diagrammatically presents the relative evolutionary associations among 25 sportive lemur species (Fig. 4).

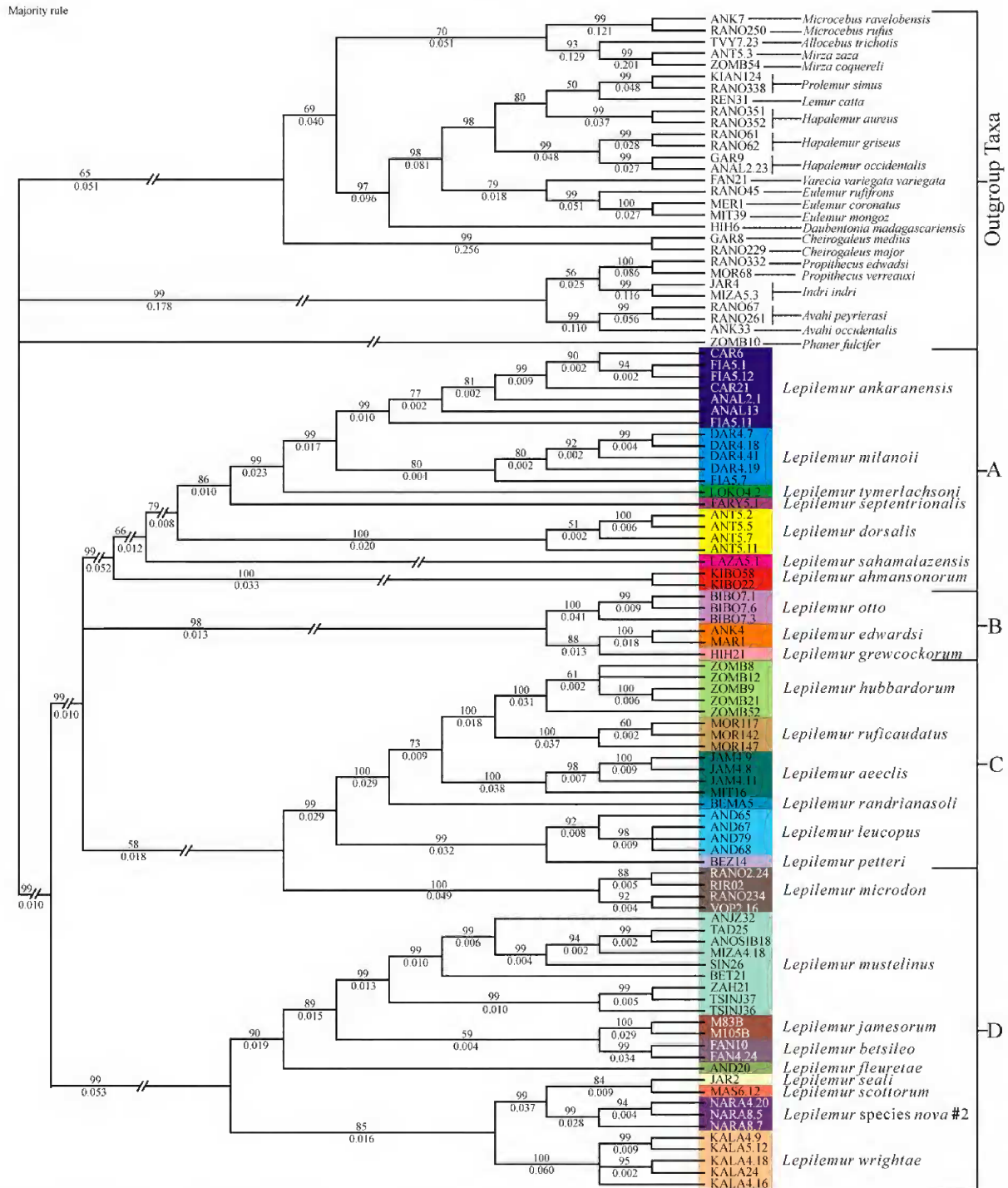


Figure 2. Phylogenetic relationships between *Lepilemur* species inferred from the maximum likelihood and Bayesian approaches for the D-loop sequence data from 73 haplotypes from the 225 *Lepilemur* individuals with 29 outgroup taxa. Numbers above the branches represent posterior probability support. Numbers below the branches represent ML values. We obtained the maximum likelihood tree (-Ln likelihood=12,404.88) from the D-loop alignment using a transition/transversion ratio of 2 ($k=3.99$). Section A consists of sportive lemurs from northern and northwestern Madagascar. Section B consists of sportive lemurs from northwestern Madagascar. Section C consists of sportive lemurs from west central and southern Madagascar. Section D consists of sportive lemurs except for *L. microdon* (associated with Section C) from eastern Madagascar. Outgroup taxonomy based on Mittermeier et al. (2008).

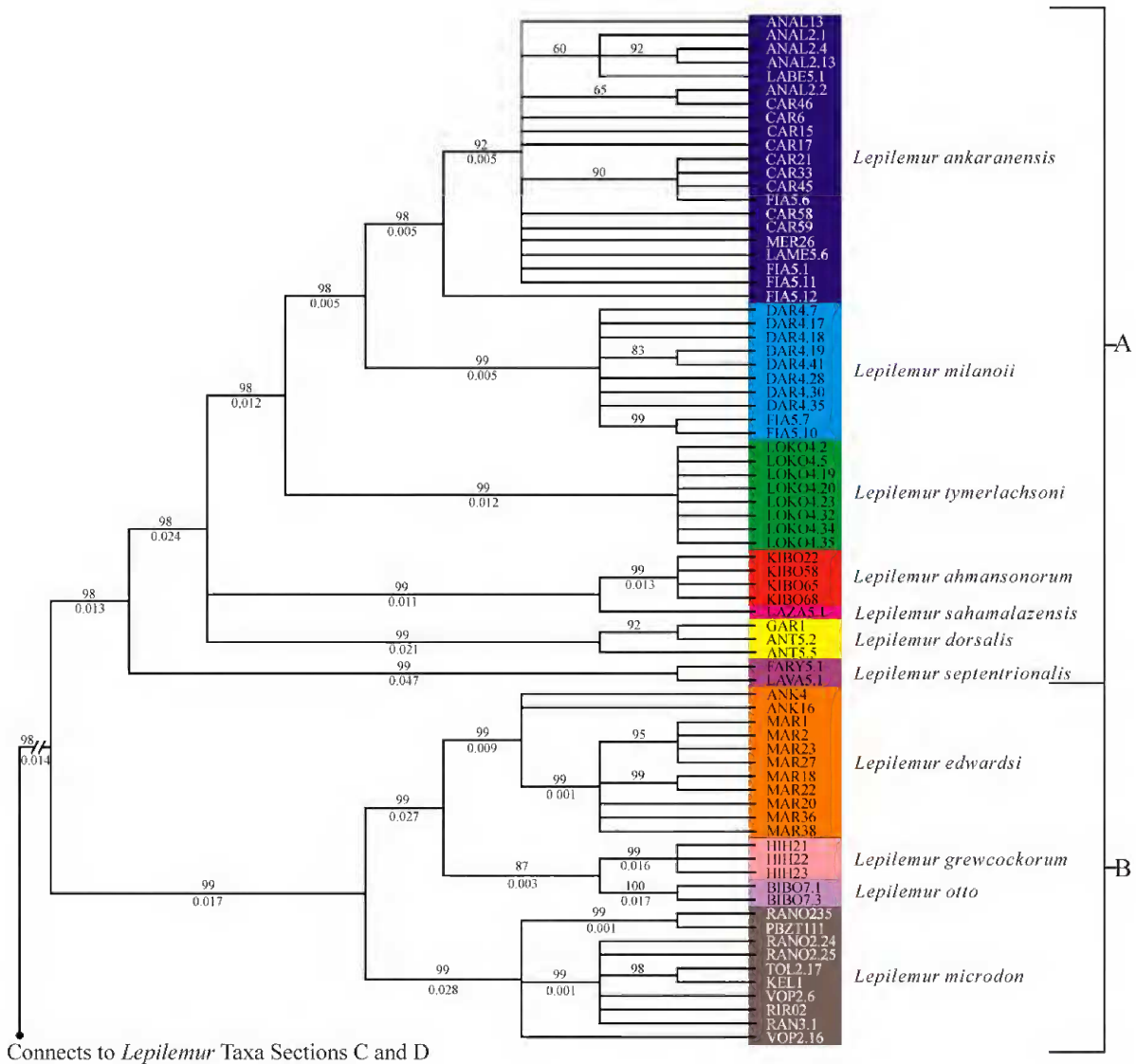


Figure 3a. Phylogenetic relationships between *Lepilemur* species inferred from the maximum likelihood and Bayesian approaches of PAST fragment sequence data from 161 haplotypes from the 225 *Lepilemur* individuals with 29 outgroup taxa. Numbers above the branches represent posterior probability support. Numbers below the branches represent ML values. We obtained the maximum likelihood tree (-Ln likelihood=38,278.20) from the PAST alignment using a transition/transversion ratio of 2 ($k=4.23$). Section A consists of sportive lemurs from northern and northwestern Madagascar. Section B consists of sportive lemurs from northwestern Madagascar.

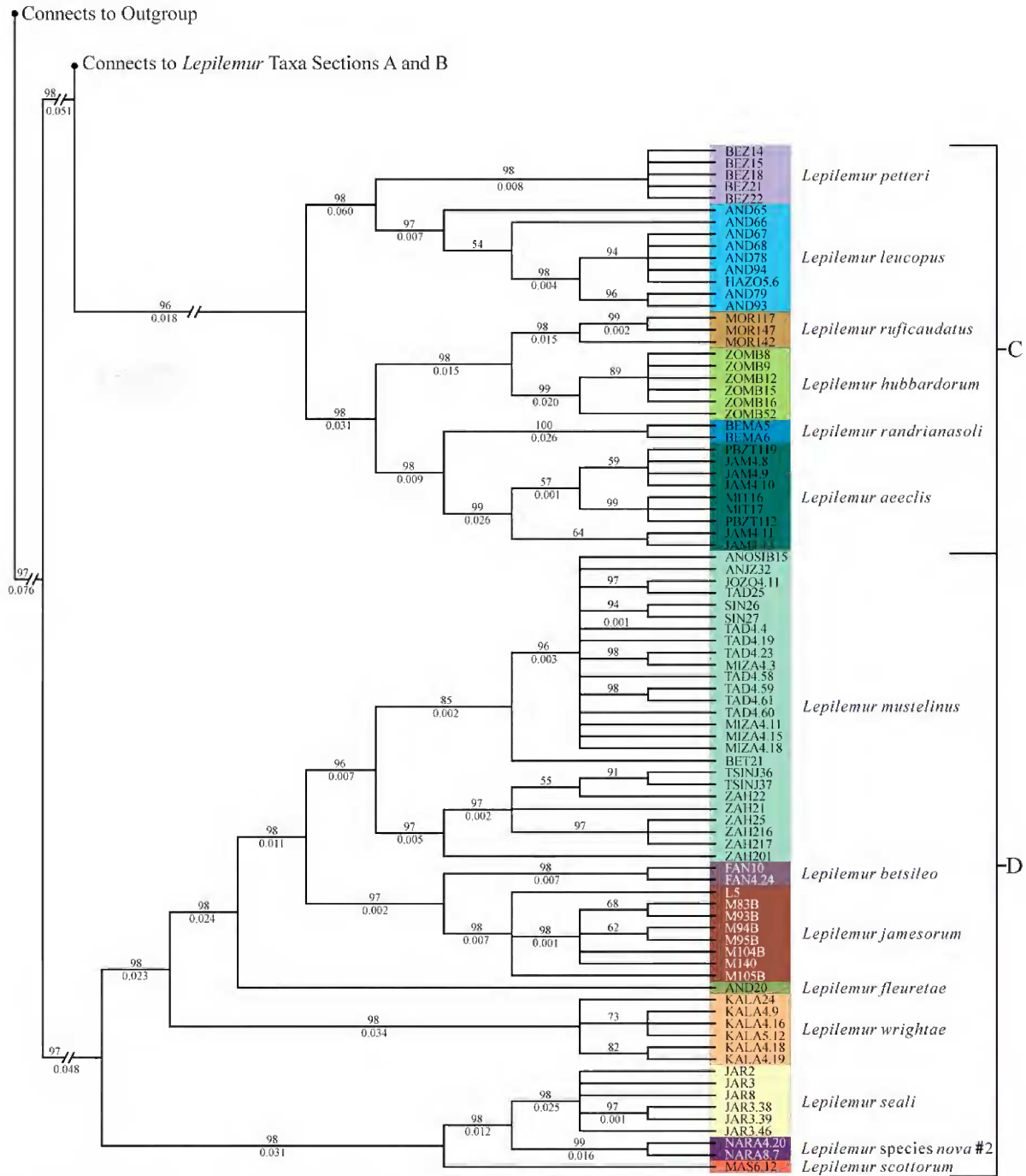


Figure 3b. Phylogenetic relationships between *Lepilemur* species inferred from the maximum likelihood and Bayesian approaches of PAST fragment sequence data from 161 haplotypes from the 225 *Lepilemur* individuals with 29 outgroup taxa. Numbers above the branches represent posterior probability support. Numbers below the branches represent ML values. Section C consists of sportive lemurs from west central and southern Madagascar. Section D consists of sportive lemurs except for *L. microdon* (associated with Section B) from eastern Madagascar.

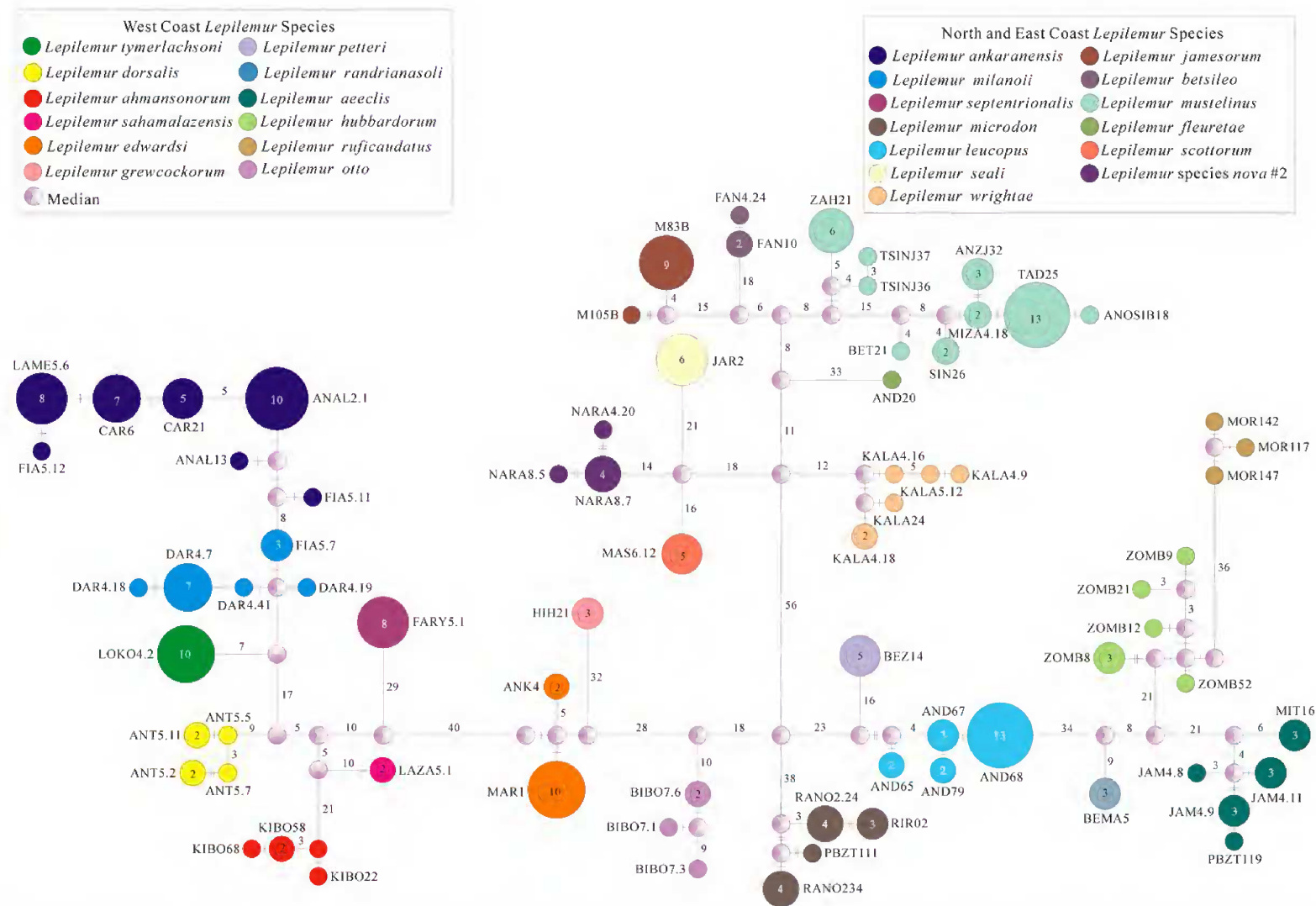


Figure 4. Minimum spanning network of *Lepilemur* haplotypes calculated using Arlequin Version 2 and Network Version 4.11. Identification numbers denote haplotypes corresponding to Table 1 (Appendix III(e)). The minimum number of mutational steps separating matriarchal lines is indicated. Nucleotide substitutions are indicated by dashes. The number of nucleotide differences in their connecting lines is indicated when there are more than two. Missing intermediates are indicated by gray circles. The size of the circles is representative of the number of individuals with matching haplotypes (circles without any number represent one individual).

DISCUSSION

Anthropogenic pressure has resulted in the fragmentation of panmictic populations, which has compelled wildlife and conservation agencies to make management priorities according to the current understanding of taxonomy, historical biogeography and present distribution of these now isolated populations (Wilmé et al. 2006; Kremen et al. 2008). Many studies have shown that molecular genetics offers a reliable and rapid method of identifying unique and/or cryptic biodiversity (e.g., Louis et al. 2006b; Craul et al. 2007, 2008). Through the analyses of accessioned and novel sample sets, we found that each described sportive lemur clusters in distinct and well-supported terminal clades, along with the undefined individuals from MNBR. With this aim, we present here a revision of the genus *Lepilemur*, concentrating on the distribution of the sportive lemur species in northeastern Madagascar.

Ganzhorn et al. (2006) described the importance of rivers as corridors referring to cytogenetic reconstructions of the genus *Lepilemur*, specifically the sister relationship of a western sportive lemur, *L. edwardsi* (Ankarafantsika National Park) and an eastern sportive lemur, *L. microdon* (Ranomafana National Park; Ishak et al. 1992). The current phylogenetic reconstructions from sequence-based data of the genus *Lepilemur* have substantiated this finding (Louis et al. 2006b; Lei et al. 2008). However, incongruence between the D-loop and PAST inferences in regard to the phylogenetic relationships of *L. microdon* to the northwestern sportive lemurs (Section B) and the west central and southern sportive lemurs (Section C) presents an alternative system of river corridor dispersal (Figs. 2-3). This finding illustrates the importance of developing and assessing novel nuclear DNA based sequence fragments to examine further the significance or function of river corridors in speciation.

The population aggregate analysis (PAA) results from the D-loop and PAST sequence fragments are presented in Tables 2A and 2B, respectively (Appendix III(a-b)). Multiple diagnostic characters distinguish each described sportive lemur, along with the undefined sportive lemur from Mananara-Nord Biosphere Reserve. *Lepilemur* species *nova* #2 had 12 diagnostic

sites for D-loop and PAST fragments combined. According to the Phylogenetic Species Concept (*sensu* Wheeler and Platnick 2000), diagnostic characters or attributes define Evolutionary Significant Units (ESUs). Several authors suggest that ESUs are equivalent to species and reflect species barriers (Cracraft 1983; Groves 2001a). *Lepilemur* species *nova* #2 had multiple molecular diagnostic sites (Tables 2A and 2B; Appendices III(a-b)) and, given this criterion, represents a distinct ESU. The continuous addition of sequence from novel samples and sites to the PAA data set will dynamically test the distinction and diagnostic ability of these characters, and, therefore, the ongoing status of this and related species.

A summary of the morphometric data for the three sportive lemur species of northeastern Madagascar, along with the proposed species, *Lepilemur* species *nova* #2, are presented in Tables 3A and 3B (detailed morphological measurements of the novel sportive lemur are available in Appendix I(b)). No extensive quantitative analyses were conducted on the morphometric data at this point (Table 3). Therefore, this morphometric information is provided as supplemental data, complementing the molecular data used to partition unique biodiversity. Nevertheless, it can be seen that the new species most resembles *L. mustelinus* in size, but has a distinctly longer tail and shorter pollex and hallux. Meanwhile, we describe the new species as follows:

***Lepilemur hollandorum*, New Species**

Lepilemur species *nova* #2 of Lei et al. (2008)

Type Specimen.—NARA4.20 (TK125726; TTU-M 109031), adult female; collected on 8 August 2004, captured at Mananara-Nord Biosphere Reserve. Material: Total genomic DNA (50ng/μl) for NARA4.20 (TK 125726), adult female, stored and curated at the Museum of Texas Tech University, Lubbock, Texas, USA. Two 2.0 mm biopsies from ear pinna, and 0.007 cc of whole blood tissues stored at Henry Doorly Zoo, Omaha, Nebraska, USA. A microchip pit tag was placed subcutaneously between the scapulae and recorded as 4556420944. NARA4.20 was collected by Edward E. Louis, Jr., Richard Randriamampionona,

Table 2A. Summary of Population Aggregate Analysis (PAA) D-loop diagnostic sites for *Lepilemur*. Refer to Appendix III(a). The locality of *Lepilemur species nova* #2 is Mananara-Nord Biosphere Reserve. *No character or attribute is available for this fragment.

| Species | Fragment Size (bp) | PAA base pair location |
|----------------------------------|--------------------|---|
| <i>Lepilemur ankaranensis</i> | 540 | * |
| <i>Lepilemur milanoii</i> | 540 | 130 |
| <i>Lepilemur tymerlachsoni</i> | 538 | * |
| <i>Lepilemur septentrionalis</i> | 536 | 33, 43, 111, 113, 249 |
| <i>Lepilemur dorsalis</i> | 540 | 536, 537 |
| <i>Lepilemur sahamalazensis</i> | 542 | * |
| <i>Lepilemur petteri</i> | 534 | * |
| <i>Lepilemur leucopus</i> | 535 | 19 |
| <i>Lepilemur ruficaudatus</i> | 535 | 103, 126, 310 |
| <i>Lepilemur hubbardorum</i> | 535 | 242, 253, 270, 302 |
| <i>Lepilemur randrianasoli</i> | 538 | 33, 272 |
| <i>Lepilemur edwardsi</i> | 545-546 | 127 |
| <i>Lepilemur grewcockorum</i> | 544 | 139, 195, 357 |
| <i>Lepilemur ahmansonorum</i> | 542 | * |
| <i>Lepilemur aeeclis</i> | 537-538 | 21 |
| <i>Lepilemur mustelinus</i> | 552-553 | * |
| <i>Lepilemur jamesorum</i> | 552 | 132 |
| <i>Lepilemur betsileo</i> | 553 | 272, 273, 286 |
| <i>Lepilemur fleuretae</i> | 550 | 10, 24, 37, 200, 287, 288, 314, 317, 330 |
| <i>Lepilemur microdon</i> | 530 | 25, 34, 107, 110, 120, 121, 123, 124, 125, 127, 137, 139, 396 |
| <i>Lepilemur wrightae</i> | 551 | 55, 58, 275, 301, 476, 493 |
| <i>Lepilemur seali</i> | 550 | 54, 159, 221 |
| <i>Lepilemur species nova</i> #2 | 550 | 87, 195, 231, 327, 475 |
| <i>Lepilemur scottorum</i> | 551 | 24, 30, 140, 187, 266 |
| <i>Lepilemur otto</i> | 545-547 | 160, 162 |

Table 2B. Summary of Population Aggregate Analysis (PAA) PAST fragment diagnostic sites for *Lepilemur*. Refer to Appendix III(b). The locality of *Lepilemur species nova #2* is Mananara-Nord Biosphere Reserve.

| Species | Fragment Size (bp) | PAA base pair location |
|----------------------------------|--------------------|--|
| <i>Lepilemur ankaranensis</i> | 2359-2360 | 364, 858, 1315, 1804 |
| <i>Lepilemur milanoii</i> | 2359 | 342, 769, 1896 |
| <i>Lepilemur tymerlachsoni</i> | 2359 | 152, 1309, 1378, 1861, 1898, 1995 |
| <i>Lepilemur septentrionalis</i> | 2360 | 44, 113, 211, 214, 274, 353, 354, 533, 551, 555, 576, 674, 734, 1103, 1174, 1231, 1347, 1399, 1448, 1492, 1550, 1582, 1603, 1630, 1777, 2144, 2146, 2363 |
| <i>Lepilemur dorsalis</i> | 2361 | 579, 717, 746, 1525, 1780, 2163, 2168, 2177, 2236 |
| <i>Lepilemur sahamalazensis</i> | 2360 | 204, 539, 737, 749, 770, 803, 1358 |
| <i>Lepilemur petteri</i> | 2360 | 337, 578, 779, 957, 1615 |
| <i>Lepilemur leucopus</i> | 2360-2361 | 220, 719, 836, 1960 |
| <i>Lepilemur ruficaudatus</i> | 2360 | 94, 127, 235, 365, 776, 1074, 1370, 1783, 1835, 1867, 1921, 2068 |
| <i>Lepilemur hubbardorum</i> | 2361 | 350, 543, 566, 629, 681, 1012, 1015, 1240, 1396, 1559, 1906, 1907, 2111 |
| <i>Lepilemur randrianasoli</i> | 2360 | 191, 699, 849, 923, 982, 1018, 1035, 1053, 1219, 1432, 1444, 1753, 1981, 1988, 2250, 2267 |
| <i>Lepilemur edwardsi</i> | 2360 | 1018, 1474, 1979 |
| <i>Lepilemur grewockorum</i> | 2360 | 406, 888, 896, 988, 1114, 1226, 1354, 1537 |
| <i>Lepilemur ahmansonorum</i> | 2360 | 46, 304, 350, 1096, 1097, 1818, 2141, 2170 |
| <i>Lepilemur aeeclis</i> | 2360 | 535, 548, 563, 581, 975, 1357, 1368, 1423, 1442, 1990, 2089, 2107 |
| <i>Lepilemur mustelinus</i> | 2358-2359 | 85 |
| <i>Lepilemur jamesorum</i> | 2359 | 2144 |
| <i>Lepilemur betsileo</i> | 2359 | 8, 1057 |
| <i>Lepilemur fleuretae</i> | 2359 | 29, 103, 269, 358, 533, 534, 546, 553, 664, 1124, 1574, 2013, 2023 |
| <i>Lepilemur microdon</i> | 2360 | 146, 510, 581, 596, 826, 829, 1171, 1954, 1991, 2077, 2164 |
| <i>Lepilemur wrightae</i> | 2359 | 55, 133, 663, 691, 871, 907, 942, 1105, 1117, 1120, 1837, 1856, 1936, 2041, 2096, 2181, 2185, 2331 |
| <i>Lepilemur seali</i> | 2360 | 124, 147, 290, 626, 665, 692, 722, 1302, 1313, 1371, 1679, 1875, 1879, 1969 |
| <i>Lepilemur species nova #2</i> | 2360 | 86, 567, 1157, 1337, 1483, 1606, 2165 |
| <i>Lepilemur scottorum</i> | 2360 | 72, 256, 1033, 1112, 1167, 1237, 1336, 1538, 1902 |
| <i>Lepilemur otto</i> | 2360 | 115, 196, 328, 379, 702, 988, 1004, 1955 |

Table 3A. Morphometric data collected from the four geographically closest species of *Lepilemur* from northeast Madagascar. (Individual morphological data available at Appendix I(b).) *Only adult individuals were included (based on body weight and canine length).

| Species Name | Common Name | *N | Weight (kg) | Head Crown (cm) | Body Length (cm) | Tail Length (cm) |
|----------------------------------|------------------------|----|-------------|-----------------|------------------|------------------|
| <i>Lepilemur mustelinus</i> | Weasel sportive lemur | 27 | 0.99±0.21 | 7.9±1.4 | 25.8±4.0 | 25.2±2.1 |
| <i>Lepilemur seali</i> | Seal's sportive lemur | 5 | 0.95±0.09 | 7.5±0.9 | 27.5±1.4 | 26.0±1.4 |
| <i>Lepilemur scottorum</i> | Scott's sportive lemur | 5 | 0.88±0.14 | 6.7±0.6 | 26.6±0.9 | 27.8±2.0 |
| <i>Lepilemur</i> species nova #2 | | 5 | 0.99±0.02 | 7.5±0.5 | 24.0±1.7 | 28.1±1.3 |

Table 3B. Morphometric data collected from the four geographically closest species of *Lepilemur* from northeast Madagascar. (Individual morphological data available online; see Appendix I(b).) *Only adult individuals were included (based on body weight and canine length).

| Species Name | Forelimb | | | | | Hindlimb | | | | |
|----------------------------------|--------------------|----------------|------------------|-------------------------|---------------------|--------------------|----------------|------------------|-------------------|-------------------|
| | Pollex Length (cm) | LD Length (cm) | Hand Length (cm) | Ulna/Radius Length (cm) | Humerus Length (cm) | Hallux Length (cm) | LD Length (cm) | Foot Length (cm) | Tibia Length (cm) | Femur Length (cm) |
| <i>Lepilemur mustelinus</i> | 2.6±0.3 | 3.6±1.4 | 6.2±0.4 | 8.0±0.7 | 6.4±0.7 | 4.8±0.7 | 2.9±0.4 | 9.1±0.4 | 9.7±0.9 | 10.9±0.9 |
| <i>Lepilemur seali</i> | 2.2±0.3 | 3.2±0.4 | 6.2±0.2 | 8.4±0.3 | 6.2±0.3 | 3.9±1.2 | 3.2±0.6 | 9.2±0.3 | 10.4±0.8 | 10.6±0.8 |
| <i>Lepilemur scottorum</i> | 2.1±0.3 | 2.9±0.2 | 5.9±0.3 | 8.4±0.4 | 6.3±0.3 | 3.5±0.2 | 2.8±0.3 | 8.3±1.3 | 9.3±2.0 | 8.7±0.5 |
| <i>Lepilemur</i> species nova #2 | 1.8±0.5 | 3.1±0.2 | 6.3±0.2 | 7.9±0.7 | 5.9±0.3 | 3.2±0.7 | 2.9±0.6 | 9.4±0.4 | 9.7±0.8 | 10.8±0.5 |

Richard Rakotonomenjanahary, Jean C. Randriamana, Justin Andrianasolo, Jean Claude Rakotoniaina, Jean Freddy Ranaivoarisoa, Boromé Ramaromilanto, Fidelis Razafimanjato, and Jean Aimé Andriamihaja on 8 August 2004.

Type Series.—Whole blood for NARA4.20 (TK125726; TTU-M 109031), adult female; NARA8.5 (TK125727; TTU-M 109032), adult male; and NARA8.7 (TK125728; TTU-M 109033), adult female; are stored and curated at the Museum of Texas Tech University. Individual measurements, e-voucher photos, and collection data are given in Appendix I(b). NARA4.20, NARA8.5, and NARA8.7 were collected by Edward E. Louis, Jr., Brandon D. Sitzmann, Richard Randriamampionona, Richard Rakotonomenjanahary, Jean C. Randriamana, Justin Andrianasolo, Jean Claude Rakotoniaina, Jean Freddy Ranaivoarisoa, Boromé Ramaromilanto, Fidelis Razafimanjato, and Jean Aimé Andriamihaja on 8 August 2004, 21 February 2008, and 21 February 2008, respectively (Appendix I(b)).

Type Locality.—Mananara-Nord Biosphere Reserve, Toamasina Province, Madagascar (approximately S16°18'22.6", E049°47'03.9").

Description.—*Lepilemur hollandorum* is a large-sized sportive lemur (0.99 kg; Table 3). The pelage on the head, along the shoulders down to the mid back is mottled reddish-gray, where the coat then transitions to a lighter grayish-brown down to the pygal region of the tail (Fig. 5; Appendix I(b)). The head has a faint dark brown to black middle dorsal stripe or inverted Y-shaped pattern. The dorsal mid-line or stripe progresses from the head to the lower half of the back. Ears protrude out and are fleshy. The ventral coat is primarily light gray, with darker undertones. The venter and face are generally gray while the neck area close to the ears and chin are lighter brown to blonde. The hands and feet are grayish-brown, and the tail is dark brown to black towards the distal end.

Diagnosis.—In the D-loop and PAST sequence fragments, *Lepilemur hollandorum* differs from its closest relatives by genetic and geographic distances, *L. seali*, *L. scottorum* and *L. mustelinus*, as follows: 7.5%±1.2% (43 informative sites), 7.0%±1.2% (39 informative sites), and 10.9%±1.4% (68 informative

sites), respectively; and 4.2%±0.4% (98 informative sites), 4.2%±0.4% (95 informative sites), and 11.0%±0.8% (253 informative sites), respectively. *Lepilemur hollandorum* has 12 diagnostic attributes (five attributes for D-loop and seven attributes for PAST fragment).

Distribution.—Currently, *L. hollandorum* is known only from Mananara-Nord Biosphere Reserve, in the Ivontaka-Sud and Verezanantsoro (Ambinanibeorana) parcels in IRS MS (Fig. 1). The Ivontaka-Sud and Verezanantsoro (Ambinanibeorana) parcels are lowland rainforest fragments. The northern boundary of *L. hollandorum* has not been defined but is expected to be either the Fahambahy or Mananara River (Craul et al. 2008). Furthermore, the lack of comprehensive samples between Zahamena National Park and Mananara-Nord Biosphere Reserve prevents definitive resolution of the southern boundary. Therefore, additional survey work is required to establish the sportive lemur biodiversity of IRS SMAS (Marotandrano and Ambatovaky Special Reserves), IRS SM, and IRS FM (Figs. 1 and 6). Additionally, IRS AAMB, IRS A, IRS AR, and IRS RFa are proposed as the distribution of *L. seali*, but additional survey work should be initiated to define the northern boundary of this sportive lemur (Figs. 1 and 6). Furthermore, the Ankavanana River is proposed as the northern boundary of *L. scottorum* (IRS MAA; Figs. 1 and 6).

Comparisons and Remarks.—*Lepilemur hollandorum* (0.99 kg) is larger in weight than *L. seali* (0.95 kg) and *L. scottorum* (0.88 kg), but it is similar in weight to *L. mustelinus* (0.99 kg; Tables 3A and 3B). Despite the geographic proximity of *L. hollandorum* to *L. mustelinus*, the genetic proximity of *L. seali*, *L. hollandorum*, *L. scottorum*, and *L. wrightae* versus that of *L. mustelinus* to the southeastern sportive lemurs provides further support for species status of *L. hollandorum* (Figs. 2-3; Appendices II(a-i)).

Etymology.—The name *hollandorum* is proposed in honor of Dick and Mary Holland for their philanthropic support for art, science, education, and research, including providing opportunities for young Malagasy scientists.

Vernacular Names.—Holland's or Mananara-Nord sportive lemur.



Figure 5. *Lepilemur hollandorum*, Holland's or Mananara-Nord sportive lemur. Photo by Edward E. Louis, Jr.

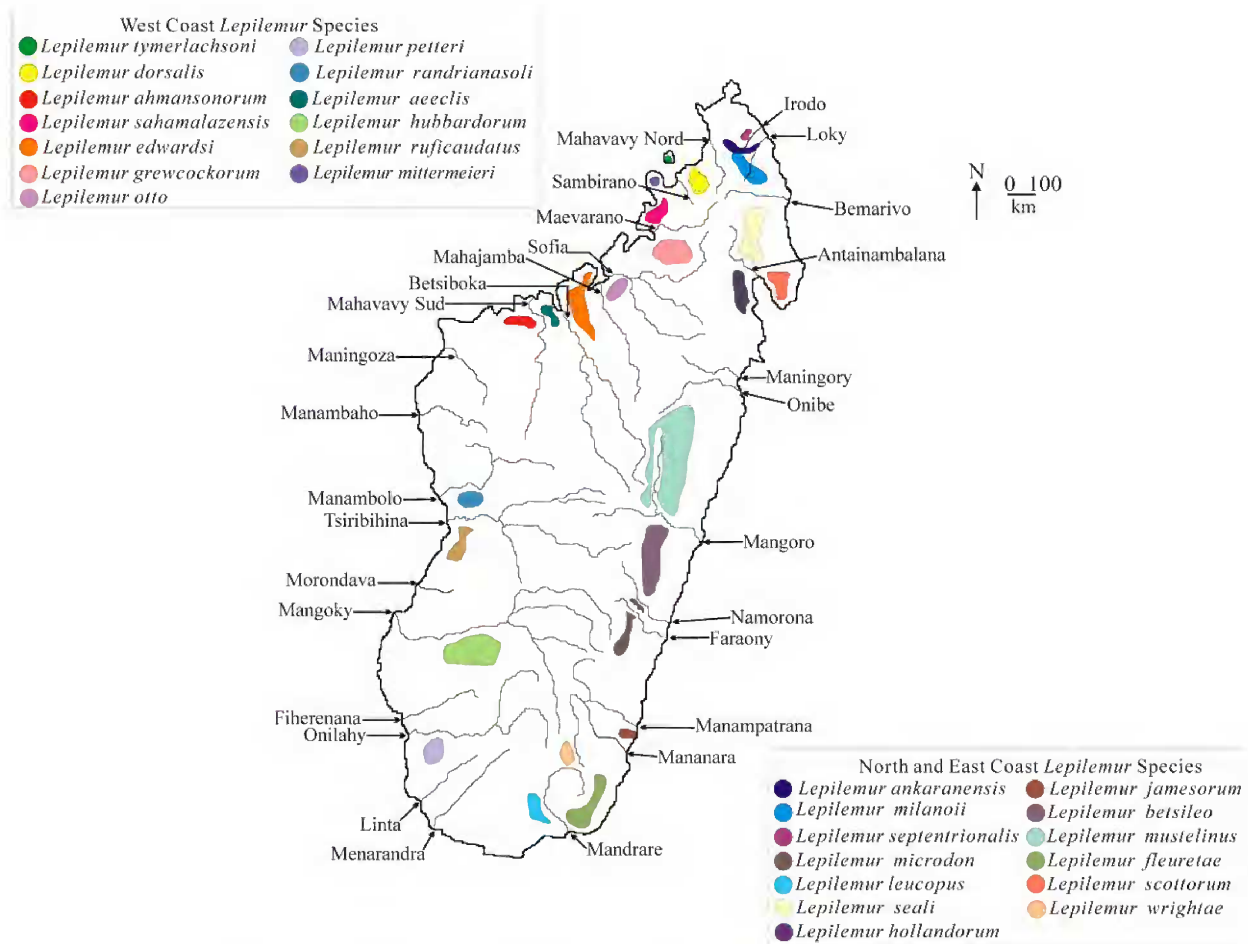


Figure 6. Revised distribution map of the sportive lemurs (genus *Lepilemur*) of Madagascar.

Despite the well-supported divergence of north-eastern sportive lemur species based on genetic characters, firm evidence for geographic isolation of these taxa is presently lacking. *Lepilemur hollandorum* may be delimited by rivers acting as barriers to dispersal although the specific rivers have not been defined (Craul et al. 2008). We propose that the Fahambahy or Mananara Rivers bound the northern distribution of the MNBR sportive lemur (Fig. 1). *Lepilemur seali* is located north of this region (IRS RFa), at least as far

south as the Fananehana River (Craul et al. 2008; Fig. 1). Furthermore, we propose the Simianona, Sandratsio, or Maningory River as the southern boundary. *L. mustelinus* is found to the south of these river systems (Fig. 1; Mittermeier et al. 2006; Louis et al. 2006b). Field surveys between each of these rivers will be needed to refine the distribution and Inter-River-System model of the sportive lemurs of northeastern Madagascar (Fig. 1).

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The following Appendices are available online at the indicated website addresses as portable document format (pdf) documents.

APPENDIX I

- (a). Appendix I(a). Table of all *Lepilemur* individual samples and corresponding information including TK number, site, original species designation, current species designation, GenBank accession number of sequence data.
- (b). Appendix I(b). Individual field data information for each novel *Lepilemur*, including morphometrics, photos, global position system, microchip data, gender, and location.

<http://www.omahazoo.com/ccr/genetics/papers/ManNordLepiAppendixI.pdf>

APPENDIX II

- (a). Appendix II(a). *Lepilemur* D-loop fragment haplotypes Bayesian analysis cladogram.
- (b). Appendix II(b). *Lepilemur* PAST fragment haplotypes Bayesian analysis cladogram.
- (c). Appendix II(c). *Lepilemur* D-loop fragment haplotypes maximum likelihood phylogram.
- (d). Appendix II(d). *Lepilemur* PAST fragment haplotypes maximum likelihood phylogram.
- (e). Appendix II(e). *Lepilemur* D-loop fragment total maximum likelihood phylogram.
- (f). Appendix II(f). *Lepilemur* D-loop fragment total Bayesian analysis cladogram.
- (g). Appendix II(g). *Lepilemur* PAST fragment total maximum likelihood phylogram.
- (h). Appendix II(h). *Lepilemur* PAST fragment total Bayesian analysis cladogram.

<http://www.omahazoo.com/ccr/genetics/papers/ManNordLepiAppendixII.pdf>

APPENDIX III

- (a). Table 1A. Diagnostic nucleotide sites from the D-loop fragment Pairwise Aggregate Analysis (PAA) of *Lepilemur*.
- (b). Table 1B. Diagnostic nucleotide sites from the PAST fragment Pairwise Aggregate Analysis (PAA) of *Lepilemur*.
- (c). Table 4A. Genetic distance matrix for D-Loop fragment sequence data for *Lepilemur* species.
- (d). Table 4B. Genetic distance matrix for PAST fragment sequence data for *Lepilemur* species.
- (e). Haplotype Table 1 (Summary of designated haplotypes for the genus *Lepilemur* from all localities for D-loop and PAST fragments).

<http://www.omahazoo.com/ccr/genetics/papers/ManNordLepiAppendixIII.pdf>